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# Oligodendrocyte–macrophage interactions *in vitro* triggered by specific antibodies

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## SUMMARY

The final pathway of myelin destruction in immune-mediated demyelination is phagocytosis by macrophages. As part of a systematic study of mechanisms of myelin–oligodendrocyte injury, we have used an *in vitro* approach to investigate interactions between rat oligodendrocytes and macrophages in order to identify the conditions under which macrophages adhere to and damage oligodendrocytes. No adherence was seen when macrophages alone were co-cultured with homologous oligodendrocytes. However, macrophage attachment to oligodendrocytes was triggered not only by antibody to the major cell-surface component galactocerebroside, but also by antibody to the quantitatively minor antigen, myelin–oligodendrocyte glycoprotein; immunocytochemical observations suggested that phagocytosis of myelin antigen also occurred. No such changes were seen in the presence of an irrelevant (anti-progesterone) antibody, or in the presence of activated complement. These results emphasize that a variety of antibodies, including those to minor myelin–oligodendrocyte antigens, may play a significant role in the development of demyelinated lesions.

## INTRODUCTION

Ultra-structural studies in human and experimental demyelinating disease have demonstrated that myelin within central nervous system lesions is ultimately phagocytosed and degraded by macrophages.[1–4] It is unlikely that macrophages adhere to and attack normal myelin, and although immune reactions have been implicated in the pathogenesis of demyelination, the precise mechanisms by which macrophages are recruited and targeted against myelin are unknown.

Oligodendrocytes synthesize myelin in the central nervous system (CNS) and continue to support this metabolically active membranous extension throughout the lifetime of each cell. Oligodendrocytes can be grown in cell culture, providing a means of investigating the response of the oligodendrocyte–myelin unit to injury and damage. In this study purified populations of rat macrophages were co-cultured with syngeneic oligodendrocytes to investigate *in vitro* mechanisms of macrophage-mediated damage. The effects of complement and antibodies on cell–cell interactions were studied in order to define the conditions under which macrophages adhere to and damage oligodendrocytes.

## MATERIALS AND METHODS

### *Cell culture*

*Oligodendrocytes* were prepared from dissociated neonatal Wistar rat optic nerves and cultured on poly-L-lysine-coated glass coverslips in Dulbecco's Modified Eagles' medium supplemented with 0·5% heat-inactivated foetal calf serum, Bottenstein Sato additives[5] and gentamicin (25 μg/ml), as described previously.[6]

*Macrophages* were prepared from adult Wistar rats using conventional methods.[7] Briefly, 30 ml sterile Earl's balanced salt solution were injected intraperitoneally into anaesthetized but otherwise untreated rats and then re-aspirated into the syringe. The resulting cell suspension was centrifuged at 0° (1500 *g* for 15 min) and the pellet resuspended and triturated (twice through a 25G needle and twice through a 27G needle) in 1 ml oligodendrocyte culture medium (see above). Viable cells were counted in the presence of trypan blue in a haemocytometer before adding defined quantities to oligodendrocyte cultures; each preparation yielded between 10 and 30 million cells/rat. After 1 hr, non-adherent cells were removed by dipping coverslips gently into 20 ml sterile culture medium in a universal container and replacing in culture wells with fresh medium, leaving only macrophages and oligodendrocytes.

### *Immunocytochemistry*

Oligodendrocytes were identified by indirect immunofluorescence, using monoclonal mouse anti-galactocerebroside (GalC)[8] and rabbit polyclonal anti-myelin basic protein antibodies (MBP);[9] prepared in our laboratory with appropriate fluorescent conjugates.

Macrophages were also identified immunocytochemically, using monoclonal antibodies OX42 (a gift from Dr D. W. Mason, University of Oxford, U.K.; culture supernatant applied to live cells at 1 : 5 dilution) and ED1 (Serotech, Bicester Oxon, U.K.; applied to cells at 20 μg/ml after fixation with 2%

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paraformaldehyde, then acetone for 5 min at 0°) followed by fluorescent antibody conjugates. OX42 is directed against the CR3 C3bi receptor and identifies macrophages and granulocytes; ED1 reacts with an as yet undefined cytoplasmic antigen and is highly specific for cells of the macrophage–monocyte line.[10,11]

*Effect of antibody or complement on co-cultures*

The effects on oligodendrocyte–macrophage interactions of two antibodies specific for oligodendrocyte surface antigens—anti-MOG;[12] kindly donated by Dr C. Linington used at 10 μg/ml, and anti-GalC (ascitic fluid diluted 1:100)—and a control anti-progesterone antibody (10 μg/ml) were investigated by preincubating oligodendrocytes in culture wells (initially containing 300 μl medium) with each antibody for 20 min, before adding between $5 \times 10^5$ and $2 \times 10^6$ macrophages; a further 1–2 ml medium were added after 1 hr.

Oligodendrocytes are known to activate complement via the classical pathway in the absence of specific antibody;[13,14] the effects of activated complement could therefore be studied independently of antibody by adding homologous serum to oligodendrocyte–macrophage co-cultures, previous experiments having indicated directly and indirectly that C3 activation occurs on oligodendrocytes in these circumstances.[13,14] Two approaches were taken: first, oligodendrocytes were exposed to sub-lethal concentrations of homologous serum (1:30) immediately before adding macrophages, in order to determine whether injured oligodendrocytes were more attractive to macrophages than intact cells. Second, larger, potentially lytic, concentrations of complement (serum at 1:5 or 1:10 dilution) were used to increase the concentration of activation products available for macrophage opsonization; direct oligodendrocyte lysis by complement membrane attack complexes in these experiments was avoided by depleting serum of C9 *in vitro* before adding to co-cultures (as described elsewhere)[13]. These concentrations of serum were selected in order to achieve concentrations of C3b which were comparable with those of immunoglobulin in the antibody experiments (10 μg/ml of anti-MOG represents approximately 70 nM, while the concentration of C3 in 1:10 diluted serum is 0·13 mg/ml, or approximately 130 nM).

In separate experiments using each of these approaches, the serum complement source was added to oligodendrocyte culture wells 2 hr after (rather than immediately before) adding macrophages.

Propidium iodide (PI; Sigma) was used as an indicator of oligodendrocyte damage.[13] This is excluded by the intact cell membrane, but once this is damaged and permeabilized, the cell nucleus becomes fluorescent as a consequence of PI intercalation with DNA.[15] PI was added to a final concentration of 100 μM; oligodendrocytes were then studied using a Zeiss inverted fluorescence microscope, and the percentage among approximately 200 cells/coverslip showing PI uptake was established.

*Electron microscopy*

Cell co-cultures on coverslips were prepared for scanning electron microscopy by fixation for 4 min at 0° in 2% paraformaldehyde, dehydration by sequential 10-min immersions in 50%, 70%, 90% and 100% ethanol, critical point drying and sputter coating with palladium gold, and then imaged using a Jeol JSM 840 A scanning electron microscope.

Immunocytochemical techniques were also used to study oligodendrocyte–macrophage interactions. Following incubation for 48–72 hr in the presence of antibody, co-cultures were fixed with 2% paraformaldehyde and double stained with monoclonal antibody ED1, to identify macrophages, and rabbit polyclonal anti-MBP antibody.

## RESULTS

### Characterization of cell preparations

Rat peritoneal cell preparations comprised highly purified populations of macrophages; a mean of 97% (SEM 2; $n = 4$) cells were ED1 positive, and similar results (98% positive; SEM 1) were obtained using OX42.

Morphologically, macrophages initially appeared as uniformly round cells of 5–8 μm diameter, but after 48 hr in culture approximately a third had adopted a flatter shape more akin to fibroblasts; both types could readily be distinguished morphologically from oligodendrocytes.

As previously reported, characterization of neonatal rat optic nerve cultures confirmed a high degree of oligodendrocyte purity; after 6 days *in vitro*, 85–90% cells were GalC-positive oligodendrocytes, 3% were astrocytes, and the remainder were large flat cells presumed to be fibroblasts.[13]

### Oligodendrocyte–macrophage interactions

In initial experiments, damage to 7-day cultures of oligodendrocytes incubated with macrophages ($10^5$–$10^6$ fresh viable cells per well) was assessed after 2, 4, 8, 24 and 48 hr using PI permeability. No PI permeabilization of either cell type was observed in these untreated co-cultures. No macrophage adhesion to oligodendrocytes was seen by light microscopy, and scanning electron microscopy confirmed the absence of any discernible cell–cell interactions in these circumstances.

Subsequent experiments determined the effect on macrophage–oligodendrocyte co-cultures of the presence of activated complement or antibody.

*Complement.* In repeated studies ($n = 6$), sub-lethal concentrations (1:30) of normal serum or 1:5 diluted C9-depleted serum, added either immediately before or 2 hr after introducing macrophages, did not cause oligodendrocyte permeabilization to PI, despite varying the number of macrophages added ($10^5$–$2 \times 10^7$). Light and electron microscopic observations repeated on three separate occasions showed that both C9-depleted serum and sub-lytic concentrations of whole serum also failed to cause macrophage adhesion to oligodendrocytes (Fig. 1).

Control experiments performed in the absence of macrophages confirmed that each batch of rat serum was lytic to oligodendrocytes as a result of complement activation, as described previously,[13,14] and that addition of purified C9 restored this cell-specific cytotoxicity to C9-depleted serum.

*Antibody.* In repeated experiments ($n \geqslant 5$ for each antibody) there was no PI permeabilization of adherent oligodendrocytes in the presence of either anti-MOG, anti-Gc or anti-progesterone antibodies. However, there did appear to be significant oligodendrocyte dislodgement from coverslips in the presence of either oligodendrocyte-specific antibody, only approximately 40% oligodendrocytes remaining attached after 48 hr compared with anti-progesterone antibody. Oligodendrocyte detachment

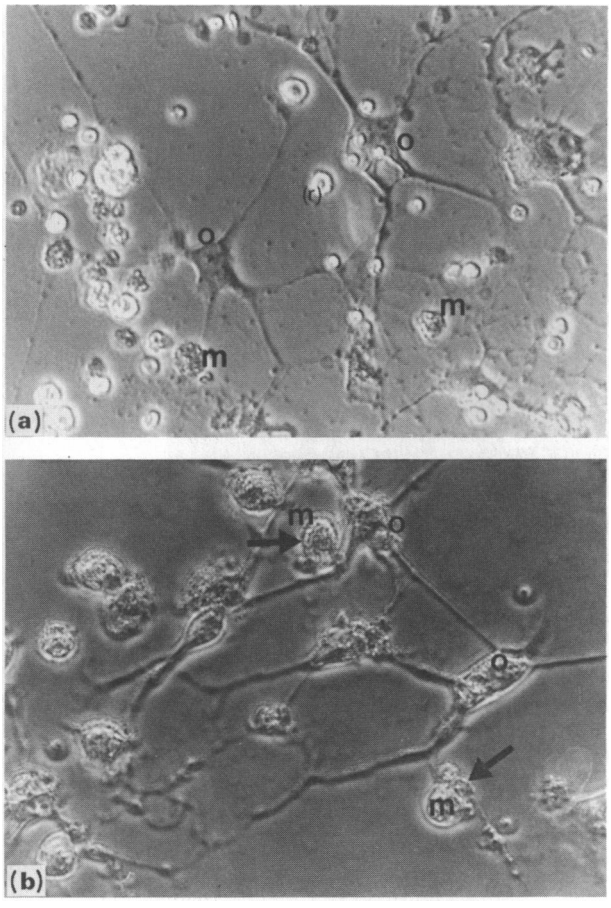


**Figure 1.** Co-cultures of oligodendrocytes (o) and macrophages (m). In the presence of 1:10 diluted homologous C9-depleted serum, without antibody, no interactions between the two cell types are observed (a), but macrophages appear adherent to oligodendrocytes in the presence of anti-MOG antibody (b). A few contaminating red cells (r) are present. Double exposure; phase contrast + PI optics; ×280.

was difficult to quantify, since it proved impossible either reliably to count the oligodendrocytes (approximately $10^4$–$10^5$) initially present on each coverslip or to identify detached PI-positive material in culture supernatants after centrifugation and examination of smeared or resuspended sediment by fluorescence microscopy.

Although no PI permeabilization was seen, phase-contrast light microscopic examination of co-cultures suggested that, following treatment with anti-GalC or anti-MOG antibody, macrophages had become attached, after approximately 24 hr, to the cell bodies and processes of those oligodendrocytes remaining on coverslips (Fig. 1). Macrophage adhesion was not observed following single or repeated doses of anti-progesterone antibody.

*Scanning electron microscopy* confirmed that, 24–48 hr after a single addition of either anti-MOG or anti-GalC antibody, macrophages adherent to approximately 50% of oligodendrocyte cell bodies and processes were clearly visible ($n > 4$ for each antibody; Fig. 2). No difference was discernible in the fine structure of the macrophage-oligodendrocyte adhesion induced by the two antibodies. No macrophage–oligodendrocyte attachment occurred in the presence of control anti-progesterone antibody ($n = 4$).

*Immunocytochemistry* in control experiments where co-cultures were treated with anti-progesterone antibody showed that discrete MBP-positive oligodendrocytes and ED1-positive macrophages were readily identifiable (Fig. 3). Approximately 10% of macrophages stained with anti-MBP antibody in co-cultures treated with anti-progesterone antibody. By contrast, in co-cultures treated with anti-MOG antibody, much larger numbers of macrophages that appeared to contain MBP were present (Fig. 3). Higher power oil immersion fluorescence microscopy (× 1000) showed granular MBP staining of macrophages mainly towards the cell circumference (Fig. 3); these granules were not visible by phase-contrast examination. MBP-positive granules were also found in macrophages which did not appear to be adherent to oligodendrocyte bodies or processes, although attachment to underlying oligodendrocyte membrane could not be excluded.

## DISCUSSION

These experiments confirm that the simple approach of adding unstimulated peritoneal rat macrophages to established cultures of homologous oligodendrocytes allows interactions between these two cell types to be investigated *in vitro*. In untreated co-cultures of normal oligodendrocytes and resting macrophages, or those treated with an irrelevant antibody (anti-progesterone) or with complement, neither cell appears much affected by the presence of the other; some macrophages later come to contain small amounts of MBP, but there is no sustained attachment of macrophages to oligodendrocytes, the latter remaining PI negative and firmly adherent to the substrate. If a single dose of either anti-GalC or anti-MOG antibody is applied, macrophages become attached to oligodendrocyte cell bodies and their processes, and granular staining for myelin antigen occurs in a pattern consistent with myelin membrane phagocytosis by macrophages; no PI permeabilization of oligodendrocytes is seen, but a proportion of oligodendrocytes become detached from the substrate; whether these subsequently become PI positive could not be determined.

These results are therefore consistent with the hypothesis that oligodendrocytes and macrophages do not ordinarily interact *in vivo*, but antibodies to oligodendrocyte surface antigens opsonize the myelin–oligodendrocyte unit, stimulating attachment and phagocytosis by macrophages through their Fc receptors. The results with control anti-progesterone antibody show that non-specific Fc receptor occupancy alone is not sufficient to allow macrophage binding; the small amount of staining for MBP within macrophages in these circumstances might be explained by natural wastage of a small proportion of oligodendrocytes in tissue culture and the known scavenging effect of macrophages when confronted with dead cells or debris.

Macrophages also possess C3b receptors, and adhesion to oligodendrocytes might also therefore be expected in the presence of activated complement. However, functional differences between macrophage C3b and Fc receptors provide a clear explanation for the absence of complement-induced oligodendrocyte–macrophage interactions.

The Fc receptor is constitutively active in resting macrophages, and Fc binding triggers both attachment to and

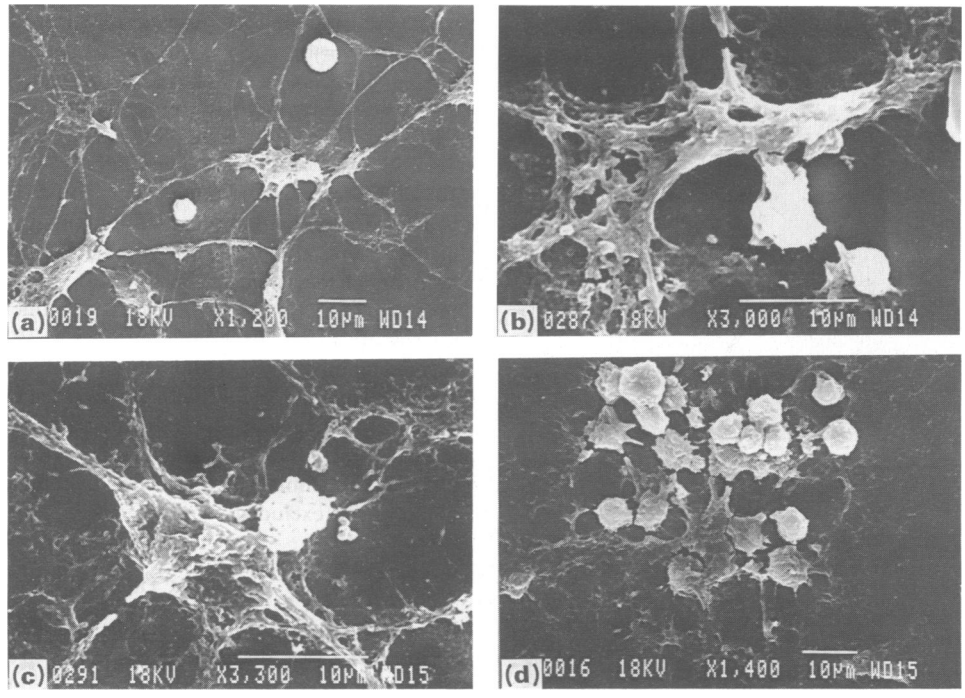


**Figure 2.** Scanning electron micrographs of oligodendrocyte–macrophage interactions. No contact occurs in the presence of irrelevant (anti-progesterone) antibody (a), but incubation with either anti-GalC (b) or anti-MOG (c) antibody triggers macrophage attachment to oligodendrocytes. Occasional oligodendrocytes show large numbers of adherent macrophages (d; anti-MOG antibody).

phagocytosis of antibody-coated targets.[16] In contrast, the resting macrophage C3b receptor is inactive and cannot initiate phagocytosis; it may attach to C3b-coated targets,[17–19] but only transiently since enzymatic degradation by serum C3b inactivator and proteases gives C3b a short half-life. The C3b receptor may be activated by lymphokines,[18] whereupon receptor occupancy mediates both adherence and phagocytosis. The rapid dissolution of the C3b:C3b-receptor link following C3b degradation would account for the absence of any discernible macrophage attachment to oligodendrocytes in the presence of activated complement.

Although we are not aware of any previous *in vitro* studies of oligodendrocytes and macrophages, interactions between macrophages and purified myelin have been investigated. Treatment of myelin with normal serum, gamma-globulin, or heat-inactivated serum, triggered macrophage adhesion; greater opsonic activity was shown by anti-myelin antiserum and its gamma-globulin fraction, and by anti-MBP and anti-GalC antisera, than by anti-myelin-associated glycoprotein (MAG) antiserum.[20] Homogenates prepared from thioglycollate-elicited rat peritoneal macrophages degraded lipids in purified rat myelin,[21] while in studies using radiolabelled myelin, Trotter *et al.* [22] found that treatment with non-immune sera and a variety of anti-myelin antibodies led to the formation of macrophage-associated radioactivity. Radiolabel attached to the surface was not distinguished from ingested material, but transmission electron microscopy showed that myelin pretreated with anti-myelin antiserum was ingested by macrophages, whereas that treated with non-immune serum simply adhered to the external macrophage surface.

However, the disruption of the normal topographical arrangement of membrane proteins during myelin extraction exposes components such as MBP which could attract macrophages but which are normally exclusively localized on the unexposed cytoplasmic face of the myelin membrane;[23,24] direct extrapolation of these results to the *in vivo* situation cannot therefore be justified. A second methodological feature complicating interpretation of these *in vitro* studies is the use in single experiments of material from different animal species—for example, bovine myelin, murine macrophages, and rabbit, mouse, human and guinea-pig sera or antisera;[20] this might also artifactually exaggerate opsonization. Previous studies have also consistently used artificially activated thioglycolate-elicited macrophages.

The present studies were designed to avoid each of these potential sources of difficulty in interpretation, and the findings may have implications for the pathogenesis of demyelinating disease. They indicate that complement activation at the oligodendrocyte surface is unlikely in the absence of specific antibody to provide a stimulus adequate for the recruitment of resident macrophages (microglia) or circulating unstimulated monocytes. While it is clearly possible that activated lymphocytes in demylelinating lesions[25] could activate macrophage C3b receptors by secreting lymphokines, the presence of surface IgG on macrophages engaged in myelin breakdown in demyelinating lesions suggest that specific antibodies are involved in myelin–oligodendrocyte opsonization *in vivo*.[26]

Immunoglobulin is synthesized within the CNS in demyelinating disease, but no common myelin–oligodendrocyte-specific antigenic determinant has been identified in cerebrospi-

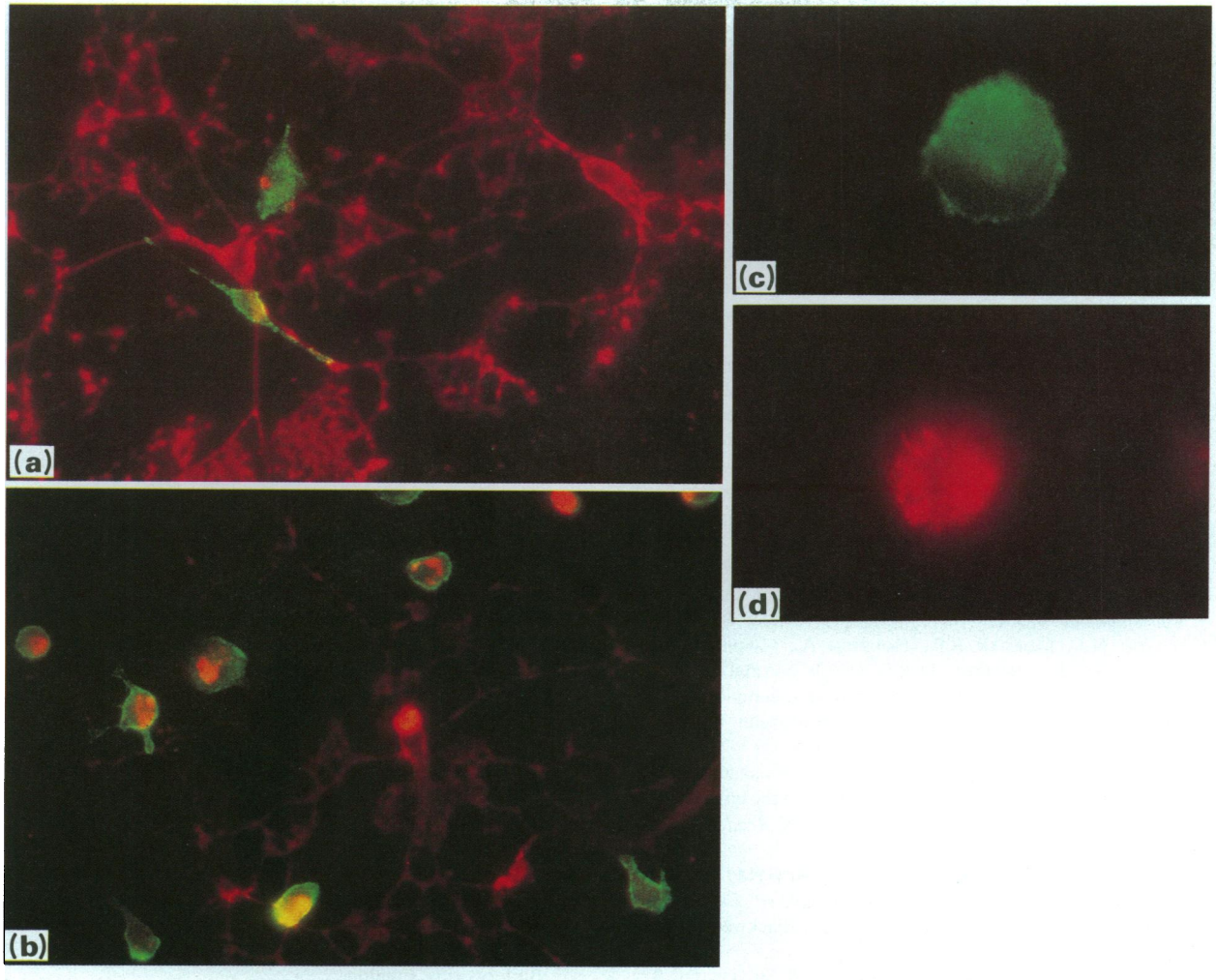


**Figure 3.** Dual-label immunofluorescence staining shows discrete MBP-positive oligodendrocytes (shown red with TRITC–anti-rabbit IgG second antibody) and ED1-positive macrophages (green following FITC–anti-mouse Ig second antibody staining) following treatment with anti-progesterone antibody (a; double exposure using fluorescein and rhodamine optics; ×260). In contrast, after incubation with anti-MOG antibody, a considerable number of macrophages also stain for intracellular MBP (b; details as for a). Higher power microscopy shows the granular pattern of MBP staining within macrophages (c; ED1 disclosed using fluorescein optics; and d, MBP using rhodamine optics; ×650).

nal fluid samples from patients;[27] conversely the *in vitro* findings reported here imply that non-specific antibody is unlikely to opsonize oligodendrocytes or myelin.

It may be relevant, however, that antibodies to the quantitatively minor antigen MOG (myelin–oligodendrocyte glycoprotein) appeared no less effective at stimulating macrophage adherence *in vitro* than those directed against the major component galactocerebroside. Conventional methods of seeking myelin-specific antibodies may lack the sensitivity necessary to detect those directed against such minor components. A novel suggestion may be made which links the *in vitro* ability of a variety of anti-oligodendrocyte antibodies to recruit and direct macrophages with the absence of a single common antigenic determinant within intrathecal antibodies in multiple sclerosis. It may be speculated that antibodies of different myelin–oligodendrocyte specificity are present in different patients, or even in the same individual at different stages during the course of the disease, and that each is capable of opsonizing myelin.

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